# TITLE OF THE DISCLOSURE

[0001] Compositions and Methods for Identifying Modulators of HAUSP

## BACKGROUND OF THE DISCLOSURE

[0002] The disclosure relates generally to the field of modulating cell survival and apoptosis.

[0003] The p53 tumor suppressor protein is a sequence-specific transcription factor that responds to a wide variety of cellular stress signals (Levine, 1997, Cell 88:323-331; Michael et al., 2003, Semin. Cancer. Biol. 13:49-58; Vogelstein et al., 2000, Nature 408:307-310). The normal function of p53 is indispensable to many cellular processes, such as cell cycle control and apoptosis. It is generally believed that p53 activity is primarily controlled at the level of post-translational modification, particularly ubiquitination (Brooks et al., 2003, Curr. Opin. Cell Biol. 15:164-171; Haupt et al., 2002, Oncogene 21:8223-8231; Yang et al., 2004, Oncogene 23:2096-2106).

[0004] In normal cells, p53 is maintained at a low level mainly through MDM2-mediated ubiquitination and subsequent degradation (Haupt et al., 1997, Nature 387:296-299; Honda et al., 1997, FEBS Lett. 420:25-27; Kubbutat et al., 1997, Nature 387:299-303). MDM2 is a RING finger E3 ubiquitin ligase (Honda et al., 2000, Oncogene 19:1473-1476) that specifically interacts with p53 through its N-terminal p53-binding domain (Leng et al., 1995, Oncogene 10:1275-1282; Chen et al., 1993, Mol. Cell. Biol. 13:4107-4114; Oliner et al., 1993, Nature 362:857-860; Kussie et al., 1996, Science 274:948-953). The C-terminal RING finger motif of MDM2 not only promotes p53 ubiquitination, but also targets MDM2 itself for autoubiquitination and subsequent degradation (Honda et al., 2000, Oncogene 19:1473-1476; Fang et al., 2000, J. Biol. Chem. 275:8945-8951). In addition, MDM2 is also a negative regulator of p53-mediated transcriptional activity (Momand et al., 1992, Cell 69:1237-1245).

[0005] The *MDM2* gene is a transcriptional target of p53, so the activation of *MDM2* gene by p53 would lead to the repression of p53 activity (Wu et al., 1993, Genes Dev. 7:1126-1132; Barak et al., 1994, Genes Dev. 8:1739-1749; Prives, 1998, Cell 95:5-8). The existence of this auto-regulatory feedback loop between MDM2 and p53 adds a complex

feature to the p53-MDM2 pathway and makes MDM2 one of the most important regulators of p53 activity.

**[0006]** The MDM2-p53 pathway is one of the most important pathways involved in p53 regulation. Previous studies imply that multiple mechanisms are involved in the stabilization of p53 through regulation of the MDM2-p53 pathway. For example, both phosphorylation and acetylation events can promote p53 stabilization. Furthermore, in response to oncogenic activation, the tumor suppressor can also stabilize p53 through the inhibition of MDM2 ubiquitin ligase activity. Besides, the profound effect of ubiquitination on the p53-MDM2 pathway clearly provides an opportunity for deubiquitination to play a role. In 2002, HAUSP was found to be a novel p53-interacting protein. HAUSP can directly deubiquitinate p53 both in vivo and in vitro, and this deubiquitination function strongly stabilizes p53 and promotes p53-dependent apoptosis.

**[0007]** As a counter measure of protein ubiquitination, protein deubiquitination has also been recognized as an important regulatory step in many cellular processes (Amerik et al., 2004, Biochim. Biophys. Acta 1695:189-207; Wing, 2003, Int. J. Biochem. Cell. Biol. 35:590-605; Kim et al., 2003, J. Biochem. (Tokyo) 134:9-18; Wilkinson, 2000, Semin. Cell. Dev. Biol. 11:141-148). Most deubiquitinating enzymes (DUBs) are cysteine proteases that specifically cleave ubiquitin from its conjugates. Growing evidence shows that DUBs can act at many different stages throughout the ubiquitin-proteasome pathway and thus have a role in maintaining the normal functions of the ubiquitin-dependent system. There are five conserved families of DUBs, of which the ubiquitin-specific processing proteases (UBPs) constitute the largest family with more than 60 members. A unique feature for UBPs is that they contain divergent N- and/or C- terminal extensions, suggesting putative substrate-recognition modules. However, only a few UBPs with specific substrates have been identified, and consequently the mechanisms by which UBPs recognize substrates remain largely unknown.

**[0008]** With hundreds of members, the UBP family of deubiquitinating enzymes has become one of the largest enzyme families known to date. For this large family of enzymes, substrate specificity has always been an important yet unresolved issue. The fact that most UBPs contain divergent sequences at their N-termini, C-termini, or both, strongly indicates that UBPs may have their own specific substrates and the divergent N-or C-terminal sequences are likely to be involved in the recognition of those substrates. Studies have also

shown that, in higher eukaryotes, UBPs are involved in the regulation of various biological processes, including eye development, cell growth, oncogenic transformation and cell cycle regulation. Despite strong evidence suggesting that UBPs may have specialized regulatory functions in multicellular organisms, only very few UBPs with specific substrates have been identified, among which HAUSP is the first mammalian example.

**[0009]** In 1997, HAUSP was first identified as a cellular interacting protein with the herpes simplex virus type I immediate early gene product, Vmw110 (also called ICP0), and was then named HAUSP (herpesvirus-associated ubiquitin-specific protease, also known as USP7). Vmw110 specifically binds to HAUSP C-terminal domain. In addition to Vmw110, another viral protein EBNA1 (Epstein-Barr virus nuclear antigen 1) was also identified to interact with HAUSP. Further studies show that the N-terminal domain of HAUSP is responsible for EBNA1 binding and an EBNA1 peptide of residues 395-450 was sufficient for binding to HAUSP. As will be mentioned later, the HAUSP N-terminal domain, which is responsible for EBNA1 binding, is also responsible for p53 recognition. The fact that EBNA1 binds to HAUSP N-terminal domain and efficiently competes off p53 peptide suggests that EBNA1 might also affect p53 function in vivo by competing for HAUSP.

**[0010]** Using an affinity-based approach, HAUSP was identified as a novel p53-interacting protein (Li et al., 2002, Nature 416:648-653; Lim et al., 2004, Int. J. Oncol. 24:357-364). HAUSP directly binds to and deubiquitinates p53 both in vivo and in vitro. Expression of HAUSP was shown to stabilize p53 in vivo and promote p53-dependent apoptosis and cell growth arrest. These observations revealed an important mechanism for p53 stabilization and identified HAUSP as the first mammalian UBP with a known substrate. More recently, HAUSP was shown to physically interact with MDM2 in a p53-independent manner (Li et al., 2004, Mol. Cell 13:879-886; Cummins et al., 2004, Cell Cycle 3:689-692; Cummins et al., 2004, Nature 428:1 p following p. 486). HAUSP can deubiquitinate MDM2 both in vivo and in vitro and is required for the stability of MDM2 in normal cells.

**[0011]** The observations that HAUSP can directly interact with and deubiquitinate both p53 and MDM2 create a conundrum: how can HAUSP stabilize p53 while at the same time stabilize MDM2 that is primarily responsible for the destruction of p53? One hypothesis has been that only one of the two proteins, p53 and MDM2, represents a physiologically

relevant target for HAUSP. Supporting this notion, increased of MDM2 was observed in HAUSP-ablated cells, which consequently resulted in the stabilization of p53. Another contrasting hypothesis has been that HAUSP can target both p53 and MDM2 for deubiquitination and thus may play a dynamic role in the p53-MDM2 pathway. In both scenarios, in order to decipher the role of HAUSP in the p53-MDM2 pathway, it is essential to understand how HAUSP specifically recognizes p53 and MDM2 and to understand the relationship of these two recognition events. This understanding was lacking in the prior art, but is described herein.

## BRIEF SUMMARY OF THE DISCLOSURE

**[0012]** The disclosure includes a description of synthetic inhibitors of HAUSP protein binding. In one embodiment, the inhibitor has a polypeptide portion that includes the sequence

**[0013]** $P^1$ - Gly - $P^3$ - Ser,

**[0014]** wherein $P^1$ is one a Glu residue and an amino acid residue having a side chain that includes a non-polar portion and wherein $P^3$ is one a Gly residue and an amino acid residue having a side chain that includes a non-polar portion. The inhibitor can be a polypeptide, a peptidomimetic, or another synthetic compound designed using the methods described herein. Such inhibitors can be used, for example in pharmaceutical compositions for treating, alleviating, inhibiting, delaying, or preventing a variety of disorders.

**[0015]** Also described is a method of inhibiting binding between HAUSP protein and a second protein with which HAUSP protein normally binds. In one embodiment, the method includes contacting the HAUSP protein with a synthetic inhibitor having a polypeptide portion that includes the sequence

**[0016]** $P^1$ - Gly - $P^3$ - Ser,

**[0017]** wherein $P^1$ is one a Glu residue and an amino acid residue having a side chain that includes a non-polar portion and wherein $P^3$ is one a Gly residue and an amino acid residue having a side chain that includes a non-polar portion. The methods described herein can be used to prolong or inhibit survival of human cells.

**[0018]** Screening methods are also described. In one embodiment, the methods described herein are used in a method of assessing the ability of a compound to inhibit

interaction between HAUSP protein and a second protein with which HAUSP protein normally interacts. This method includes steps of

[0019] - covalently linking the compound with a portion of HAUSP protein that includes residues 53-196 of human HAUSP protein to form a linked product,

[0020] - crystallizing the linked product, and

[0021] - assessing the crystal structure of the crystallized linked product.

[0022] Interference of the compound portion of the linked product with a region of the HAUSP protein portion of the linked product with which the second protein normally binds indicates that the compound can inhibit binding between HAUSP protein and the second protein.

[0023] This disclosure also describes crystallizable products useful for assessing binding of a compound with HAUSP protein. Such products include a first polypeptide portion including residues 53-196 of human HAUSP protein linked with the compound by way of a sterically flexible linker.

[0024] The disclosure describes inhibitors which modulate interaction of the TRAF-like domain of HAUSP with proteins with which HAUSP normally binds; inhibitors which modulate interaction of ubiquitin with HAUSP; and inhibitors which modulate the isopeptidase activity of HAUSP.

## BRIEF SUMMARY OF THE SEVERAL VIEWS OF THE DRAWINGS

[0025] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0026] Figure 1 comprises Figures 1A and 1B. Figure 1A is a diagram which identifies the N-terminal domain of HAUSP as the primary p53 binding motif. Various purified recombinant HAUSP fragments were individually incubated with a large p53 fragment (residue 94-393) and then subjected to gel-filtration analysis. Figure 1 B is a diagram which identifies a p53 fragment that is necessary and sufficient for HAUSP binding. p53 peptides with various length and boundaries were purified individually and were tested for binding with the HAUSP N-terminal domain (residues 53-208) by gel-filtration.

[0027] Figure 2 is a schematic representation of the domain structure of human MDM2 protein. Full-length MDM2 protein contains four major domains (I -IV). The boundary residues for each domain are indicated.

[0028] Figure 3 comprises Figures 3A and 3B. Figure 3A is a diagram which identifies the N-terminal domain of HAUSP as the primary MDM2 binding motif. Various purified recombinant HAUSP domain constructs were individually incubated with a large fragment of Mdm2 (residues 170-423) and then subjected to a gel-filtration analysis. Figure 3B is a diagram which identifies an MDM2 fragment including the acidic region (residues 208-289) that is sufficient for HAUSP recognition.

[0029] Figure 4 is an image of the structure of HAUSP N-terminal domain. The HAUSP N-terminal TRAF-like domain adopts an eight-stranded antiparallel beta sandwich structure, with strands (beta)1, (beta)5 and (beta)6, and (beta)8 in one sheet and (beta)2, (beta)3, (beta)4, and (beta)7 in the other. The overall structure of the HAUSP TRAF-like domain resembles the TRAF-C domain of the TRAF family of proteins. Figure 4A is a color image that depicts the structure of the HAUSP TRAF-like domain in a ribbon diagram (left portion of the figure) and a surface representation (right portion of the figure). Secondary structural elements in the ribbon diagram and the putative substrate-binding groove in the surface representation are labeled. Figure 4B is alignment of the sequences HAUSP TRAF-like domain with other TRAF family members. Conserved residues are highlighted in yellow. HAUSP residues that interact with p53 through hydrogen bonds and van der Waals contacts are identified by green triangles and green squares, respectively. HAUSP residues that interact with MDM2 through hydrogen bonds and van der Waals contacts are indicated by red triangles and red squares, respectively. Conserved residues that are involved in binding to peptides in other TRAF family proteins but not in HAUSP are colored red and highlighted in purple. The ribbon and cylinder diagram above the sequences indicates secondary structures in HAUSP protein.

[0030] Figure 5 depicts the structural basis of p53 recognition by HAUSP. Figure 5A is a diagram that indicates the identity of a short peptide fragment in p53 (residues 359-362) as the HAUSP-binding element. Various p53 fragments were individually incubated with HAUSP protein (residues 53-206), their interactions were examined by gel filtration, and their ability to interact is indicated by + (interacts) or - (does not interact). Figure 5B-1 is a color image that shows the overall structure of the HAUSP TRAF-like domain bound to p53

peptide in a surface representation. Figure 5B-2 is a color ribbon diagram showing the structure of HAUSP bound with p53 peptide (left portion of the figure) and a comparison of the free and p53-bound TRAF-like domain (right portion of the figure). The comparison indicates that binding by the p53 peptide does not induce any significant conformational changes in HAUSP. Figure 5C-1 and 5C-2 are a pair of color images that provide a stereo view of the specific interactions between p53 and HAUSP. Hydrogen bonds are represented by red dashed lines. All interacting residues are labeled.

**[0031]** Figure 6 illustrates the regions of HAUSP and MDM2 proteins that interact in a stable HAUSP-MDM2 complex. Figure 6A is a diagram that illustrates that the TRAF-like domain of HAUSP is responsible for binding to MDM2. Various HAUSP fragments were individually incubated with MDM2 protein (residues 170-423), their interactions were examined by gel filtration, and their ability to interact is indicated by + (interacts) or - (does not interact). Figure 6B is a diagram that illustrates a HAUSP-binding element in MDM2. Various MDM2 fragments were individually incubated with HAUSP TRAF-like domain (residues 53-206), their interactions were examined by gel filtration, and their ability to interact is indicated by + (interacts) or - (does not interact).

**[0032]** Figure 7 includes Figures 7A and 7B. Figure 7A is a color image that shows the overall structure of the HAUSP TRAF-like domain bound to MDM2 peptide in a ribbon diagram (left portion of the figure) and in a surface representation (right portion of the figure). The important MDM2 residues are highlighted in yellow. Figures 7B-1 and 7B-2 are a pair of color images that provide a stereo view of the specific interactions between MDM2 and HAUSP. These interactions are more extensive than those between p53 and HAUSP. Hydrogen bonds are represented by red dashed lines. All interacting residues are labeled.

**[0033]** Figure 8 is a structural comparison of p53, MDM2, and EBNA1 peptide binding by HAUSP. Figure 8A is a color image showing MDM2 peptide (red) bound to the same surface groove as the p53 peptide (magenta). Residues from MDM2 and p53 are shown in yellow and green, respectively. Figure 8B-1 is a color image which shows superposition of three HAUSP-binding peptides derived from MDM2 (red), p53 (magenta), and EBNA1 (green) on a surface representation of HAUSP. The HAUSP TRAF-like domain is shown in a transparent surface representation, with critical residues shown in brown. Figure 8B-2 is an enlargement of a portion of Figure 8B-1, as indicated. Figure 8C is a structural

alignment of HAUSP-binding peptides p53, MDM2, and EBNA1, each shown bound to a transparent surface representation of the HAUSP surface groove. Figure 8D shows the consensus tetrapeptide sequence of the three peptides.

[0034] Figure 9 illustrates structural implications of the observations described herein for an extended HAUSP fragment. Figure 9A is a color ribbon diagram showing the structure of a HAUSP fragment (residues 53-560) that contains both the substrate-binding (green) and the catalytic domains. Binding sites for ubiquitin and substrate are indicated. The linker sequences between these two domains have high temperature factors and are flexible in the crystals described herein. Figure 9B is a color image of a structure-based model showing a surface representation of HAUSP bound to a ubiquitinated MDM2 protein. Only one ubiquitin moiety and the MDM2 peptide are shown in this figure.

## DETAILED DESCRIPTION

[0035] The disclosure relates to interactions between human HAUSP protein and other proteins with which HAUSP protein is known to interact. Such interactions have physiological significance in apoptosis and survival of cells. This disclosure includes substantial new information regarding the mechanism and chemistry by means of which HAUSP interacts with other proteins. The information in the disclosure can be used to make compositions for modulating such interactions, to assess the pharmacological activity of candidate compounds, to affect survival of cells, and for other purposes described herein.

[0036] The application relates to the following six sets of atomic coordinates included in the Protein Data Bank (Berman et al., 2000, The Protein Data Bank. Nucl. Acids Res. 235-242): 2F1W, 2F1X, 2F1Y, 2F1Z, 1NB8, and 1NBF.

[0037] Definitions

[0038] As used herein, each of the following terms has the meaning associated with it in this section.

[0039] A "synthetic" inhibitor is a compound which does not exist in nature in an isolated form and is made by the interaction of man with precursor materials, regardless of whether those precursor materials exist in nature. By way of example, a synthetic polypeptide can be one made using individual amino acid residues and a solid-state peptide

synthesizing apparatus or it can be a portion of a naturally-occurring protein that is cleaved by a reagent with which the protein is contacted.

[0040] In this disclosure, standard three-letter amino acid residue codes are used. By way of example, the code "Ala" specifies alanine and the code "Glu" specifies glutamic acid. Standard single-letter amino acid residue codes are also used. By way of example, the code "A" specifies alanine, and the code "E" specifies glutamic acid.

[0041] Human HAUSP protein is the protein having the amino acid sequence described in the National Center for Biotechnology Information (NCBI) database record having accession number Q93009 (Swissprot).

[0042] Human MDM2 protein is the protein having the amino acid sequence described in the NCBI database record having accession number NP_002383.

[0043] Human p53 protein is the protein having the amino acid sequence described in the NCBI database record having accession number NP_000537.

[0044] Epstein-Barr virus nuclear antigen 1 (EBNA1) protein is the protein having the amino acid sequence described in the NCBI database record having accession number P03211.

[0045] Description

[0046] In this disclosure, we report the biochemical and structural basis of the recognition of p53 and MDM2 by HAUSP. We show that both p53 and MDM2 bind to, in a mutually exclusive manner, the N-terminal TRAF-like domain of HAUSP. MDM2 exhibits a much higher binding affinity, and efficiently out-competes excess p53 for binding to HAUSP in a competition assay.

[0047] We localized the HAUSP-binding elements to short peptide fragments in p53 and in MDM2 and determined the crystal structures of HAUSP TRAF-like domain bound to such peptides. These structures reveal the molecular basis for the differential binding of p53 and MDM2 to HAUSP, which identifies a consensus peptide recognition sequence for HAUSP. A polypeptide having or including that sequence can be used as a modulator of binding between HAUSP and one or both of p53 and MDM2, as can peptides, peptide analogs, and peptidomimetics based on that structure.

[0048] The information described herein can be used for investigation and design of chemical structures for modulating binding between HAUSP and one or both of p53 and

MDM2 and thereby modulating the p53-MDM2-HAUSP ubiquitination / deubiquitination pathway. These findings have important ramifications for understanding the role of HAUSP in the p53-MDM2 pathway.

[0049] Inhibitors of HAUSP Protein Binding

[0050] This disclosure includes synthetic inhibitors of HAUSP protein binding (i.e., binding of HAUSP protein with other proteins such as MDM2, p53, and EBNA1). These inhibitors are based on the portions of HAUSP protein that are described herein as interacting with one of these proteins, on the portions of MDM2, p53, and EBNA1 proteins that are described herein as interacting with HAUSP protein, or on a combination of the two.

[0051] In one embodiment, the inhibitor is based on a consensus sequence described herein for proteins that bind with the TRAF-like domain of HAUSP. Such inhibitors have a polypeptide portion that includes the sequence

[0052] $P^1$ - Gly - $P^3$ - Ser.

[0053] In this sequence, $P^1$ is either a Glu residue or an amino acid residue having a side chain that has a non-polar portion (i.e., a residue with a non-polar side chain or a residue, such as Glu, which includes an aliphatic portion, such as the methylene moiety of the Glu side chain) and $P^3$ is either a Gly residue or an amino acid residue having a side chain that has a non-polar portion. Preferably, $P^3$ is an amino acid residue having a non-polar side chain. The inhibitor can be a polypeptide, a polypeptide that is capped on one or both ends with a moiety that inhibits or prevents degradation of the peptide, or a polypeptide having one or more non-naturally-occurring amino acid residues incorporated therein (e.g., for the purpose of inhibiting or preventing proteolytic degradation of the peptide). The inhibitor can also be a peptidomimetic compound which exhibits interactions similar to those between the TRAF-like domain of HAUSP and either p53 or MDM2 as described herein. Design and manufacture of peptidomimetic compounds is known in the art, and sufficient guidance to make and use peptidomimetics of the type described herein can be found, for example, in co-pending U.S. patent application serial number 09/965,967. Such inhibitors bind with HAUSP protein and prevent binding of proteins that normally bind with the TRAF-like domain of HAUSP in the absence of the inhibitor.

[0054] The polypeptide portion preferably includes at least four amino acid residues, and preferably does not include more than about ten residues, although longer inhibitors can be used. A skilled artisan in this field is able to design peptidomimetic compounds and other compounds which exhibit relevant portions of the polypeptide sequences disclosed herein. Candidate compounds can be screened (e.g., using the assay methods described herein) to identify the affinity with which they bind with HAUSP protein. In general, the greater the binding affinity of a candidate compound, the more effective an inhibitor it will be of HAUSP binding with a second protein, at least to the extent that the compound interferes with binding between HAUSP protein and the second protein. Such interference can include binding of the compound with an amino acid side chain moiety involved in binding between HAUSP and the second protein, chemical modification of such a moiety, alteration of the secondary or tertiary structure of HAUSP protein, steric hindrance of the site of second protein binding with HAUSP, and other phenomena known in the art.

[0055] In one embodiment, the inhibitor structure is selected based on the interactions observed in the crystal structure of the HAUSP TRAF-like domain bound to the p53 and/or MDM2 peptides. Examples include peptides and peptidomimetic compounds interacting with relevant HAUSP amino acid residues (e.g., Phe118, Ile154, Trp165, and Phe167) through van der Waals contacts and residues (e.g., Arg104, Arg152, and Asp164) through polar interactions, as observed in the crystal structures described herein.

[0056] In one embodiment, fragments of human p53 protein can be used to inhibit HAUSP protein binding. Examples of suitable fragments are those that includes residues 359-368, 357-367 of human p53 protein, or 351-382 of human p53 protein. In another embodiment, fragments of EBNA1 protein are used. Examples of suitable fragments are those that includes residues 441-450 or 422-450 of EBNA1 protein. In yet another embodiment, fragments of human MDM2 protein are used as HAUSP protein binding inhibitors. Examples of suitable fragments are those that includes residues 223-232, 210-244, or 208-289 of human MDM2 protein.

[0057] The polypeptide portion of the inhibitor can be linked with a moiety that enhances binding between the inhibitor and HAUSP protein. The identity of the moiety is not critical. It can be a portion of a protein (the same protein from which the sequence of the polypeptide portion is derived or a different protein) that normally interacts with HAUSP protein. The moiety can be a non-peptide compound that binds with HAUSP. In

another embodiment, the moiety can be an antibody or antibody-fragment that binds HAUSP.

**[0058]** The inhibitor can be linked with a detectable label, a radionuclide, or substantially any other moiety that does not interfere with binding between the polypeptide portion and HAUSP protein.

**[0059]** In another embodiment, the inhibitor has a polypeptide portion that is based on the amino acid sequence of a portion of HAUSP protein that is involved in binding of a second protein. By way of example, HAUSP is known to exhibit binding with p53, MDM2, and EBNA1 proteins. The portions of HAUSP protein involved in such binding are described herein, and include residues 53-196 of human HAUSP protein. An inhibitor according to this embodiment should include at least this portion of HAUSP protein, which approximately corresponds to the N-terminal TRAF-like domain of HAUSP. The polypeptide region can include additional HAUSP residues as well. By way of example, the region can include residues 53-208 of HAUSP. Such inhibitors bind with proteins that normally bind with the TRAF-like domain of HAUSP in the absence of the inhibitor and prevent binding of such proteins with HAUSP.

**[0060]** It is described herein that the affinity of binding between MDM2 and HAUSP proteins can be decreased by altering the amino acid residue identity of one or more of HAUSP residues 152, 162, 165, and 168. Furthermore, the affinity of binding between p53 and HAUSP proteins can be decreased by altering the amino acid residue identity of one or more of HAUSP residues 164, 165, and 167. By altering these residues (or others that are apparent from the information provided herein or upon crystallographic analysis of binding between HAUSP and another protein using the analytical techniques described herein), the binding affinity of the inhibitor can be modulated such that the inhibitor will exhibit binding specificity different than native HAUSP protein.

**[0061]** By way of example, alteration of HAUSP residues 152, 162, and 168 can be expected to decrease the affinity of HAUSP (residues 53-197) for binding with MDM2 protein. The altered residues can be selected to have relatively little effect on binding of the HAUSP fragment with p53. Such a fragment can be expected to bind MDM2 with lower affinity than native HAUSP protein, indicating that the fragment's avidity for p53 binding vis a vis MDM2 binding should be greater than that of native HAUSP. Similarly, alteration of HAUSP residues 164 and 167 can be expected to decrease the affinity of HAUSP

(residues 53-197) for binding with p53 protein more so than the affinity of the HAUSP fragment for binding MDM2. Such a fragment can be expected to bind MDM2 with greater avidity for MDM2 binding vis a vis p53 binding than native HAUSP. This latter fragment can be used in a system containing native HAUSP, MDM2, and p53 to preferentially bind MDM2 with the fragment, thereby enhancing the likelihood of binding between HAUSP and p53. Such a fragment could be expected to increase deubiquitination of p53, enhance cellular p53 levels, and decrease the likelihood of survival of the cell, for example. Such a fragment can increase self-ubiquitination and subsequent degradation of MDM2 protein.

**[0062]** Although the inhibitors described herein are discussed with reference to human HAUSP, MDM2, and p53 proteins and Epstein-Barr virus EBNA1 protein, a skilled artisan recognizes that non-human (and non-EBV) analogues of these proteins can be used in analogous ways with little, if any, experimentation required.

**[0063]** The inhibitors described herein can be administered to a cell or tissue to affect the p53-MDM2 pathway in the cell or in cells of the tissue. Inhibition of binding between p53 and HAUSP can be expected to enhanced degradation of p53 by proteasome-mediated and other pathways, leading to enhanced survival of cells. Inhibition of binding between MDM2 and HAUSP can be expected to enhance deubiquitination of p53, leading to more likely progression of a cell through the cell cycle and into apoptosis. Inhibition of binding of all proteins with the TRAF-like domain of HAUSP can be expected to lead to enhanced survival of cells, just as in HAUSP-ablated cells described by others. Selection of the type of HAUSP-protein binding inhibition is desired depends on the state of the cells and the desired result; nonetheless, such selection is within the ken of the skilled artisan in view of the information provided herein.

**[0064]** Inhibitors such as those described herein can be suspended in a pharmaceutically acceptable carrier and administered to a body location at which modulation of HAUSP-protein binding affinity is needed or desired. For tissues to which administration of a protein fragment described herein is not expected to be practical (e.g., where the fragment must be transported across the cytoplasmic membrane of a cell), a nucleic acid encoding such a fragment can be administered instead. A variety of suitable vectors (e.g., virus vectors and other vectors suitable for delivering a gene to the interior of a mammalian cell are known in the art, and substantially any of these vectors can be used to deliver an expression vector encoding a protein fragment described herein to a cell.

[0065] Inhibition of HAUSP-Protein Binding

[0066] Any of the inhibitors described herein can be used to inhibit binding of HAUSP protein with one or more proteins with which HAUSP normally (i.e., in the absence of the inhibitor) binds, or to substantially prevent such binding. Several physiologically significant events are known to be affected by levels of ubiquitinated and non-ubiquitinated p53 protein and by levels of ubiquitinated and non-ubiquitinated MDM2 protein. HAUSP is also known to bind (and actually or potentially deubiquitinate) other proteins, such as EBNA1. These events can be modulated by modulating HAUSP-protein binding using the methods described herein.

[0067] A physiological event of significant medical interest is survival of cancerous and other diseased cells. p53 Protein can induce normal (non-diseased) cells to undergo apoptosis rather than becoming diseased cells.

[0068] For example, in many cancers, defects in p53 function inhibit apoptosis of the cell, leading to cancer cell survival and proliferation. A defect in p53 function can be brought about by insufficient cellular p53 levels, and p53 levels can be affected by ubiquitination of p53 by MDM2. MDM2 also auto-ubiquitinates itself. Ubiquitinated p53 and MDM2 are degraded in cells by a proteasome-mediated pathway and possibly by other pathways. HAUSP can deubiquitinate both p53 and MDM2, but appears to exhibit greater affinity for MDM2. Although the method may not be fully understood, normal levels of HAUSP protein appear to enhance MDM2 degradation and increase cellular p53 levels. Using the inhibitors and methods described herein, levels of non-ubiquitinated p53 in cancer cells can be increased by inhibiting HAUSP binding with MDM2. Such inhibitors can therefore be used to induce cancer cells to undergo apoptosis, thereby alleviating, inhibiting, or preventing tumors.

[0069] Further by way of example, survival of some virus-infected cells appears to rely on suppression of normal p53 levels. The inhibitors described herein can be used to induce apoptosis in virus-infected cells. Inhibitors which suppress cellular p53 levels (e.g., by preferentially inhibiting binding between p53 and HAUSP) can be used to inhibit apoptosis in cells in a patient afflicted with a disorder characterized by an inappropriately high rate or extent of apoptosis of cells of one or more types. An example of such a disorder is acquired

immune deficiency syndrome (AIDS), which is characterized by excessive cell death of lymphocytes.

[0070] Screening Methods

[0071] This disclosure includes a method of assessing the ability of a compound to inhibit interaction between HAUSP protein and a second protein with which HAUSP protein normally interacts. Several specific embodiments of the method are described in the examples. The method involves covalently linking a compound with a portion of HAUSP protein that includes at least residues 68-196 (i.e., the N-terminal TRAF-like domain) of human HAUSP protein. This linked product is crystallized and its crystal structure is determined. From the crystal structure, it can be determined whether the compound interferes with a region of the HAUSP protein portion of the linked product with which the second protein normally binds. If so, then that is an indication that the compound can inhibit binding between HAUSP protein and the second protein. Furthermore, the compound can be used as a lead compound for design and development of additional compounds that can be used as inhibitors. Such design can be directed by the crystallographic analysis of the compound bound to the HAUSP protein to which it is linked.

[0072] The identity of the linker by which the compound is linked with the portion of HAUSP protein by way of a polypeptide linker is not critical. The linker should offer the compound sufficient steric freedom that it can bind with the HAUSP protein portion in a manner that is not significantly affected by the presence of the linker. That is, binding between the free (i.e., non-linked) compound and HAUSP should be essentially identical to binding between the compound and the HAUSP protein portion to which it is linked.

[0073] The three-dimensional structures of HAUSP, p53, MDM2, and EBNA1 disclosed herein can be used in computerized rational drug design (i.e., molecular modeling and molecule-molecule interaction modeling) methods to identify candidate compounds that bind with interaction portions of one or more of these proteins. A variety of computerized drug design programs capable of modeling the interaction of a candidate compound with, for example, atomic coordinates described herein, are known in the art, and the operation of such programs is within the ken of the ordinary artisan in the field of rational drug design. Such methods can be used to design compounds that interact with the TRAF-like domain of

HAUSP or portions of a HAUSP-binding protein (e.g., MDM2, p53, or EBNA1) which interact with HAUSP.

**[0074]** Candidate compounds can be designed based on the structure of a known portion of one of the proteins described herein. By way of example, adenine nucleotide analogs can be designed based on the individual sequences corresponding to the consensus sequence for binding of HAUSP with MDM2, EBNA1, and p53 described herein for the purpose of designing an analog that will interact with the TRAF-like domain of HAUSP. Alternatively, the interaction of the HAUSP TRAF-like domain with compounds in a library of known or modeled compounds can be assessed using the same rational drug design software. Iterative design methodologies can be employed, whereby a later generation of candidate compounds can be designed based on modeling data obtained with an earlier generation of candidate compounds (e.g., by modifying the structure of an earlier-generation compound for which the modeling software indicates strong TRAF-like region binding affinity).

**[0075]** A compound identified by rational drug design methods as likely exhibiting desirable interaction with HAUSP or a HAUSP-binding protein can thereafter be synthesized or purchased, and the compound's ability to interact with the protein in a desirable manner can be assessed experimentally. For example, compounds which are intended to modulate binding of HAUSP with a second protein can be assessed using any of the methods described herein.

**[0076]** Examples

**[0077]** The subject matter of this disclosure is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only, and the subject matter is not limited to these Examples, but rather encompasses all variations which are evident as a result of the teaching provided herein.

**[0078]** Example 1

**[0079]** HAUSP-Substrate Binding

**[0080]** The results of experiments described in this example demonstrate that HAUSP recognizes multiple substrates by way of binding mediated by its N-terminal domain.

**[0081]** The physiological role of HAUSP in the p53-MDM2 pathway is complicated because HAUSP exhibits a robust deubiquitinating activity toward MDM2. Just like p53,

MDM2 can also be efficiently deubiquitinated both in vitro and in vivo by HAUSP, and reduction of HAUSP by transient RNAi leads to a decreased half-life in MDM2. This observation suggests that inactivating HAUSP may have important application in anti-cancer therapies, as it will down-regulate MDM2.

**[0082]** HAUSP is an important regulatory protein in cells because HAUSP can deubiquitinate both p53 and MDM2, and thus may has an important regulatory role in the p53-MDM2 pathway.

**[0083]** The results of the experiments presented in this example are now described.

**[0084]** HAUSP recognizes the C terminus of p53

**[0085]** p53 contains an N-terminal transactivation domain (residues 15-30), a central DNA-binding core domain (residues 94-292), a tetramerization domain (residues 326-355), and a C-terminal regulatory domain (residues 356-393). The N-terminal domain (residues 1-248) of HAUSP is sufficient for binding to p53. To further define the domain boundary, we generated a series of deletion variants of HAUSP, and expressed and purified these recombinant proteins. We examined their ability to interact with p53 by gel filtration. Based on gel-filtration results, a protease-resistant fragment (residues 53-208) of HAUSP was found to be both necessary and sufficient for stable interactions with p53, while neither the core domain (residues 208-560) nor the C-terminal domain (residues 560-1102) formed a stable complex with p53. These domains are shown diagrammatically in Figure 1A.

**[0086]** To identify the minimal sequence requirement in p53, we generated a number of deletion variants and assayed their interaction with a HAUSP fragment (residues 53-208) using gel filtration. Neither the DNA-binding core domain nor the oligomerization domain of p53 was found to be required for formation of a stable complex with HAUSP. In contrast, a C-terminal 32-residue peptide of p53 (residues 351-382) was found to be sufficient for the interaction with HAUSP. In particular, a short II-residue peptide stretch (residues 357-367) of p53 plays a critical role in binding to HAUSP as removal of this sequence from p53 (residues 325-356) crippled its interactions with HAUSP. This information is shown diagrammatically in Figure 1B.

**[0087]** These analyses demonstrated that the N-terminal domain (residues 53-208) of HAUSP stably interacts with a minimal C-terminal peptide (residues 357-382) of p53.

Supporting this conclusion, the N-terminal domain of HAUSP (residues 58-196) was found to share significant homology (up to 32% sequence identity) to the TRAF (TNF receptor-associated factor) domain, a known peptide-binding motif.

**[0088]** HAUSP binds to a short peptide motif of EBNA1

**[0089]** Full-length EBNA1 protein contains 641 amino acids. The identified functional domains of EBNA1 include a long N-terminal Gly-Ala repeat (GAr), a nuclear localization signal, a dimerization domain, and a DNA-binding domain. The GAr in EBNA1 spans a sequence of 238 amino acids (residue 91-328). It helps to block antigen presentation through inhibition of the ubiquitin-dependent proteolysis of EBNA1. A 56 amino-acid region (residues 395-450) in EBNA1 has been shown to be both necessary and sufficient to bind to HAUSP. To further define the HAUSP-interacting sequence in EBNA1, we first generated two deletion variants for the previously identified HAUSP-interacting region (residues 395-450), with one retaining the N-terminal half sequence and the other retaining the C-terminal half sequence. Both deletion variants were purified to homogeneity in their GST-tagged form, and were reloaded to GS4B columns to generate EBNA1 affinity columns. The purified HAUSP N-terminal domain protein (residue 53-206), which was previously shown to interact with EBNA1, was loaded onto the EBNA1 affinity columns and was subjected to extensive washing afterwards. The binding of EBNA1 deletion variants to HAUSP were assayed through their ability to retain HAUSP N-terminal domain protein on the affinity column. The EBNA1 affinity chromatography results indicated that the N-terminal half of EBNA1 (395-422) does not bind to HAUSP N-terminal domain, while the C-terminal half sequence (residues 422-450) is both necessary and sufficient to bind to HAUSP N-terminal domain.

**[0090]** The results derived from EBNA1 affinity chromatography were further confirmed by gel-filtration chromatography experiments. According to the gel-filtration results, the EBNA1 (residues 395-422) peptide did not form a stable complex with the HAUSP N-terminal domain in solution, and was eluted separately from HAUSP N-terminal domain on gel-filtration. In contrast, the EBNA1 (residues 422-450) formed a stable complex with the HAUSP N-terminal domain, and the complex was eluted from a gel-filtration column one fraction earlier that HAUSP N-terminal domain by itself.

[0091] To further delineate the minimal HAUSP-binding sequence in EBNA1, we carried out another round of EBNA1 affinity chromatography to test the HAUSP binding ability of a series of deletion variants within the previously identified HAUSP binding sequence (residues 422-450). In order to avoid potential steric interference from the GST moiety, a flexible linker of 35 amino acids was added in between GST and EBNA1 peptide for each GST-fusion EBNA1 construct. Aliquots of loading samples, wash and eluates were run on SDS-PAGE.

[0092] According to the second-round EBNA1 affinity chromatography results, no HAUSP N-terminal domain protein was bound to GST-EBNA1 (residues 422-442), indicating that this part of the sequence may not be involved in HAUSP binding. In contrast, a substantial amount of HAUSP N-terminal domain protein was detected in the eluate from GST-EBNA1 (residues 441-450) column, indicating that this is the most critical sequence for HAUSP binding. A faint band of HAUSP N-terminal domain was also seen in the eluate sample from GST-EBNA1 (residues 446-450), which suggests that there is also binding between HAUSP and EBNA1 (residues 446-450). However, compared to the binding between HAUSP and EBNA1 (residues 441-450), EBNA1 (residues 446-450) binds to HAUSP much less tightly, indicating that further truncation of residues 441-445 removed part of the HAUSP binding sequence.

[0093] Combining the data from both affinity chromatography and gel-filtration, a minimal sequence of 10 amino acids in EBNA1 (resides 441-450) was shown to have an essential role for binding to HAUSP N-terminal domain.

[0094] HAUSP recognizes a central acidic domain of MDM2

[0095] Full-length human MDM2 includes 491 amino acid residues. According to the sequence alignment of MDM2 from different species, full-length MDM2 can be divided into four major conserved regions, as shown in Figure 2. Region I is at the N terminus of MDM2, including about 90 amino acids without obvious sequence similarity to known domain identities. This region includes the p53-binding domain. Region II is a highly acidic region with 62% sequence homology across species. This region has been shown to be involved in the binding with multiple proteins, including p14ARF (an inhibitor of MDM2) and L5 (which is a component of the large ribosomal subunit). Region III is a potential zinc finger domain with 58% sequence homology. The exact function of this

domain still remains unclear. Region IV is a highly conserved RING finger domain with 83% sequence homology. This domain is responsible for the E3 ubiquitin ligase activity of MDM2. A short basic sequence between region I and region II with a potential nuclear localization signal (NSL in Figure 2) is also highly conserved.

[0096] To study recognition between MDM2 and HAUSP, we generated a series of deletion constructs for both MDM2 and HAUSP, expressed and purified these recombinant proteins, and tested their ability to interact with each other using a gel-filtration-based assay. According to gel-filtration results, just as in the case of p53 interaction assay, the N-terminal domain of HAUSP was both necessary and sufficient for stable interactions with MDM2. Neither the core domain (208-560) nor the C-terminal domain was involved in the binding with MDM2 (as shown in Figure 3A). For MDM2, an 82 amino acid sequence (208-289) in the acidic region was sufficient to bind to HAUSP N-terminal domain (as shown in Figure 3B).

[0097] The p53 peptide (residues 351-382) can form a stable complex with the HAUSP N-terminal domain in solution. However, in the presence of MDM2, the binding between p53 and HAUSP was completely abolished. In the gel-filtration binding assay for HAUSP, MDM2 and p53, MDM2 (residues 208-289) efficiently competed off p53 peptide (residues 351-382), and formed a stable complex with HAUSP N-terminal domain (residues 208-289). The currently identified HAUSP recognition motif of MDM2 includes the central acidic domain of MDM2. This central acidic domain of MDM2 contains 37% acidic residues in mouse and human.

[0098] It has been reported that this acidic region of MDM2 also interacts specifically with L5, a component of the large ribosomal subunit, and thus is implicated in ribosome biosynthesis or in translational regulation. In addition to L5 binding, the central acidic region of MDM2 is essential for ARF binding. ARF (p14ARF and p19 ARF of human and mouse, respectively) is a small basic protein encoded by the INK4a locus, which also encodes the cyclin-dependent kinase inhibitor p16INK4a. Soon after its identification, ARF was found to interact with MDM2 and to block MDM2-mediated ubiquitination of p53. A truncated human MDM2 containing residues 208-491 was able to interact with human ARF, and deletion of residues 222-437 completely abolished its binding to ARF. Further deletion analysis has shown that an acidic domain overlapping region (residues 210-244) of human MDM2 is most critical for the ARF binding activity. The description herein that the acidic

domain is also involved in the recognition with HAUSP adds an additional function to this domain.

[0099] HAUSP N-terminal domain adopts a TRAF-like structure

[0100] In addition to the biochemical analyses of the binding between HAUSP and its substrates, a structural approach was used to further study substrate recognition by HAUSP. Given the fact that the N-terminal domain of HAUSP is involved in the recognition of three out of the four currently identified HAUSP-interacting proteins, the structure of the HAUSP N-terminal domain alone was the first focus. BLAST search results for HAUSP N-terminal domain showed that the HAUSP N-terminal domain shares 34% sequence identity with the TRAF domain (TD) of human TRAF2. Tumor necrosis factor (TNF) receptor-associated factors (TRAFs) are a family of adapter proteins that were first identified for their ability to bind to TNF family receptors (TNFRs). TRAFs can regulate the functions of the TNFR superfamily by linking the cytosolic domain of those receptors to downstream regulators such as protein kinases or ubiquitin ligases. Previously, six members of the TRAF family were identified in humans and mice, and two TRAFs have been identified in Drosophila. All known TRAFs share a highly conserved region near their C-terminus, which is referred to as the "TRAF domain" (TD). The TRAF domain represents a novel protein fold of an anti-parallel (beta)-sandwich structure and it usually consists of a sequence of about 150 amino acids. It is known that TRAFs regulate the function of TNF family receptors primarily by binding to the specific peptidyl motifs in the cytosolic domains of the receptors. The functional specificity of TRAFs arises from the different peptide motifs they recognize.

[0101] To elucidate the mechanism of substrate recognition by HAUSP, we crystallized the N-terminal domain of HAUSP (residues 53-206) and determined its structure at 1.6 Angstrom resolution using multi-wavelength anomalous dispersion. Data from that crystallographic analysis is presented in Table 1. Consistent with sequence analysis, the HAUSP N-terminal domain adopts a TRAF-like structure, as shown in Figure 4. It forms an eight-stranded anti-parallel (beta)-sandwich structure, with strands (beta)1, (beta)5, (beta)6, and (beta)8 in one sheet and (beta)2, (beta)3, (beta)4, and (beta)7 in the other.

Table 1. Summary of Crystallographic Analysis

| Data set | Native (HAUSP) | SeMet λ1 (peak) | SeMet λ2 (inflection) | SeMet λ3 (remote) |
|---|---|---|---|---|
| Beamline | X25 | X12C | X12C | X12C |
| Wavelength (Angstrom) | 1.1 | 0.9794 | 0.9797 | 0.95 |
| Resolution (Angstroms) | 1.65 | 2.40 | 2.40 | 2.40 |
| Unique reflections | 19,080 | 5801 | 5768 | 5667 |
| Data Redundancy | 6.5 | 7.1 | 7.0 | 7.0 |
| Completeness, % | 98.8 | 90.1 | 89.5 | 87.5 |
| (Outer shell) | (92.5) | (48.6) | (47.5) | (40.4) |
| I/σ (Outer shell) | 36.8 (9.72) | 15.3 (2.94) | 14.4 (2.81) | 13.4 (2.27) |
| $R_{sym}$ (Outer shell) | 0.047 (0.19) | 0.107 (0.31) | 0.099 (0.29) | 0.115 (0.40) |
| Anomal. Ciff. (%) | n/a | 7.0 | 6.5 | 7.2 |
| $R_{Cullis}$ | | 0.8 | 0.71 | 0.72 |
| Phasing power (centric/acentric) | | 0.55/0.39 | 1.16/0.66 | 0.99/0.49 |
| Overall Figure of Merit (20-2.6 Angstroms) | 0.55 | | | |
| Refinement | HAUSP | | | |
| Resolution (Angstroms) | 500-1.65 | | | |
| Reflections (\|F\|>0) | 17,311 | | | |
| All atoms (solvent) | 1,335 (174) | | | |
| $R_{cryst}/R_{free}$ (%) | 19.4/21.2 | | | |
| Rmsd bond length (Angstrom) | 0.009A | | | |
| Rmsd bond angle (deg) | 1.56 | | | |
| Ramachandran Plot | | | | |
| Most favored (%) | 86.9 | | | |
| Additionally allowed (%) | 13.1 | | | |
| Generously allowed (%) | 0 | | | |
| Disallowed (%) | 0 | | | |

$R_{sym} = \Sigma_h\Sigma_i \mid I_{h,i} - I_h \mid / \Sigma_h\Sigma_i I_{h,i}$, where $I_h$ is the mean intensity of the $i$ observations of symmetry related reflections of $h$. $R_{cryst} = \sum \mid F_{obs} - F_{calc} \mid / \sum F_{obs}$, where $F_{obs} = F_P$, and $F_{calc}$ is the calculated protein structure factor from the atomic model ($R_{free}$ was calculated with 10% of the reflections). Phasing power = $[(F_{H(calc)}^2 / (F_{PH(obs)} - F_{PH(calc)})^2]^{1/2}$, where $F_{PH(obs)}$ and $F_{PH(calc)}$ are the observed and calculated derivative structure factors, respectively. $R_{Cullis} = \sum \| F_{PH} \pm F_P \mid - F_{H(calc)} \mid / \sum \mid F_{PH} - F_P \mid$, where $F_{H(calc)}$ is the calculated heavy atom structure factor. Figure of Merit = $<\sum P(\alpha) \exp(i\alpha)/\sum P(\alpha)>$, where $P(\alpha)$ is the probability distribution for the phase α. RMSD (root-mean-square deviation) in bond lengths and angles are the deviations from ideal values.

[0102] The topology and overall structure of HAUSP N-terminal domain closely resemble those of the TRAF domain structure. The HAUSP N-terminal domain can be superimposed on the TRAF domain of human TRAF2 structure with an rmsd of 1.48 Angstroms for 101 aligned backbone C(alpha) atoms. There is a shallow surface depression in the middle portion of one side of the (beta)-sandwich structure. In TRAF domains, this surface crevice is the region responsible for peptide recognition. In agreement with this feature, the binding assay results described herein indicate that all three substrates known to interact with the HAUSP N-terminal domain use a peptidyl motif for the interaction.

[0103] The following conclusions can be drawn from the results of the experiments described in this example.

[0104] Since its identification in 1997, HAUSP has been shown to interact with at least four proteins, each of which has very important regulatory functions in the cell. There are multiple reasons to study the recognition mechanism between HAUSP and its substrates. One arises from the fact that, previously, the issue of substrate specificity of the whole UBP family deubiquitinating enzymes was elusive and not well studied. Even though there have been hypotheses claiming that the divergent sequences at the N or C terminus of UBPs endow high substrate specificity, no well-studied example had yet been presented. Being one of the few UBPs with substrates identified, HAUSP was a good target for the studies of the substrate recognition mechanism of the UBP family enzymes, as described herein. In addition, the fact that all the identified HAUSP-interacting proteins are important regulators in various biological processes makes the study of substrate recognition by HAUSP significant.

[0105] Using gel-filtration, we first identified a protease-resistant fragment of HAUSP N-terminal domain (residues 53-208) to be both necessary and sufficient for p53 binding. In a similar approach, we also identified a C-terminal peptide sequence of p53 as necessary and sufficient for binding to the N-terminal p53-recognition motif of HAUSP. The minimal peptide (residues 357-382) included as few as 26 amino acids, but it contains five of the six putative ubiquitination sites. The extensive overlap of these multiple ubiquitination sites with the HAUSP binding site imposes important structural constraints on HAUSP. First, the HAUSP p53-recognition domain must be in close proximity to the active site cleft of the

catalytic domain. Second, to accommodate these multiple ubiquitin attachment sites, the connection between the HAUSP catalytic core domain and the p53 binding domain must be flexible in order to position each ubiquitin-p53 linkage within the active site for nucleophilic attack by the catalytic Cys. This organization is likely to be important for efficient deubiquitination of p53 in vivo.

**[0106]** The recognition between HAUSP and EBNA1 was previously characterized by others. According to literature, EBNA1 binds to the N-terminal domain of HAUSP, the same domain where p53 was shown herein to bind. A 46 amino acid sequence (residues 395-450) of EBNA1 was shown to be both necessary and sufficient for HAUSP recognition. In our study, we used a combined approach of both EBNA1 affinity chromatography and gel-filtration to further define the minimal sequence requirement of EBNA1 for HAUSP binding. Based on our results, a short peptide motif of 10 amino acids (residues 441-450) in EBNA1 has an important role for HAUSP binding. Because further deletion of the peptide severely reduced the binding affinity between EBNA1 and HAUSP, this may be the minimum sequence required for HAUSP recognition. The fact that EBNA1 and p53 interact with the same domain of HAUSP raised the possibility that EBNA1 might interfere with binding between p53 and HAUSP, and the experiments described herein indicated this is the case. EBNA1 binds to the N-terminal domain of HAUSP almost 10 times more strongly than does p53, and the HAUSP-EBNA1 complex formation strongly inhibited the ability of p53 to bind with HAUSP.

**[0107]** Recognition between p53 and HAUSP has been shown to have an important role in stabilizing p53 and thereby inhibiting cell cycle progression and inducing apoptosis. The facts that EBNA1 and p53 interact with the same domain of HAUSP and that EBNA1 recognition strongly inhibits p53 binding raised the possibility that EBNA1 might interfere with cellular processes in host cells by disrupting the HAUSP-dependent deubiquitination of p53. Under this hypothesis, by destabilizing p53, EBNA1 would be expected to promote cell cycle progression and prevent apoptosis, which could be important for the host cell immortalization. Epstein-Barr virus efficiently immortalizes cells during its latent infectious cycle, and this process involves a few different Epstein-Barr virus latency proteins. It still remains unclear whether EBNA1 plays a direct role in host cell immortalization, but the findings reported by others that transgenic mice expressing EBNA1 have a tendency to develop B-cell lymphomas suggest that EBNA1 may play a role in those

processes. The results disclosed herein indicate that EBNA1 can contribute to host cell immortalization by Epstein-Barr virus through sequestering HAUSP, thereby destabilizing p53. HAUSP-sequestering agents based on the structure of the portion of EBNA1 that interacts with HAUSP are therefore candidates for agents for prolonging cell lifespan.

**[0108]** Beside p53 and EBNA1, we also identified that the N-terminal domain of HAUSP is responsible for MDM2 binding. Due to the difficulty of expressing full-length MDM2, a long fragment of MDM2 (residues 170-432) was used to identify the MDM2-binding region in HAUSP. By gel-filtration assays, we showed that neither the catalytic core domain nor the C-terminal is able to bind to the long fragment of MDM2. The HAUSP-binding region of MDM2 was localized to the central acidic region (residues 208-289). Our binding assay results also showed that, like EBNA1, MDM2 can efficiently compete off p53 peptide from binding to HAUSP. It has been previously demonstrated that HAUSP can efficiently deubiquitinate p53 and MDM2, both in vivo and in vitro. Based on these findings, a dynamic role of HAUSP in the p53-MDM2 pathway was proposed, which claims that a kind of substrate balance for HAUSP is achieved under physiological conditions: under certain conditions, HAUSP may exclusively deubiquitinate p53, while under other conditions, MDM2 may be the major substrate for HAUSP. Our finding that MDM2 can form a stable complex with HAUSP, and that this complex formation efficiently abolishes p53 binding to HAUSP, strongly argues that in vitro MDM2 is a preferred substrate for HAUSP. It has been shown that MDM2 uses multiple mechanisms to regulate p53 functions, including targeting p53 for ubiquitination and sequestering p53 transactivation activity. The results described herein indicate that, in addition to the previously identified regulatory mechanisms, MDM2 may also be able to destabilize p53 through sequestering HAUSP's deubiquitinating activity on p53.

**[0109]** The HAUSP-binding motif that we identified in MDM2 includes a sequence motif (residues 210-244) that has been shown to be essential for binding to ARF. ARF protein was previously shown to be a negative regulator of MDM2. ARF can function to stabilize p53 by blocking the E3 ubiquitin ligase activity of MDM2. Our finding that the ARF binding motif in MDM2 overlaps the HAUSP-binding region indicates an interplay network between MDM2, HAUSP, and ARF. Agents based on the sequence of ARF are therefore candidate compounds for inhibiting MDM2-HAUSP interactions.

[0110] As a further effort to study the substrate recognition mechanism of the N-terminal domain of HAUSP, the crystal structure of the HAUSP N-terminal domain was solved. The HAUSP N-terminal domain adopts a nine-stranded antiparallel (beta)-sandwich structure, which closely resembles the TRAF domain (TD) architecture, as shown in Figure 4. In the HAUSP N-terminal structure, there is a shallow surface concave at one side of the (beta)-sandwich. In TRAF structures, a similar surface depression is involved in the recognition of specific peptides. This feature strongly supports our conclusion from biochemical binding assays that the nature of the recognition between HAUSP and its substrates is peptide-protein interaction.

[0111] Example 2

[0112] Structural Basis of Competitive Recognition of p53 and MDM2 by HAUSP/USP7: Implications for the Regulation of the p53/MDM2 Pathway

[0113] HAUSP/USP7, a deubiquitinating enzyme of the UBP family, specifically deubiquitinates both p53 and MDM2. HAUSP therefore has an important role in the p53-MDM2 pathway. The results of experiments described in this example demonstrate that p53 and MDM2 specifically recognize the N-terminal TRAF-like domain of HAUSP in a mutually exclusive manner. HAUSP preferentially forms a stable HAUSP-MDM2 complex even in the presence of excess p53. The HAUSP-binding elements were mapped to a peptide fragment in the carboxyl-terminus of p53 and to a short peptide region preceding the acidic domain of MDM2. The crystal structures of the HAUSP TRAF-like domain in complex with p53 and MDM2 peptides, determined at 2.3 and 1.7 Angstrom resolution, respectively, reveal that the MDM2 peptide recognizes the same surface groove in HAUSP as that by p53 but mediates more extensive interactions. Structural comparison led to the identification of a consensus peptide recognition sequence by HAUSP. These results, together with the structure of a combined substrate-binding and deubiquitination domain of HAUSP, provide important insights into regulation of the p53-MDM2 pathway by HAUSP.

[0114] The methods used in the experiments presented in this example are now described.

[0115] Protein Preparation.

[0116] All constructs were generated using a standard PCR-based cloning strategy. The HAUSP TRAF-like domain (residues 53-206) and the HAUSP long fragment (residues 53-560) were cloned into pGEX-2T (Pharmacia), and were overexpressed in *Escherichia coli* strain BL21(DE3) as N-terminally GST-tagged proteins. Seleno-Met-substituted HAUSP TRAF-like domain (residues 53-206) was expressed in *E. coli* B834(DE3) (Novagen) in M9 minimal medium supplemented with 50 milligrams per liter selenomethionine. To generate the HAUSP-p53 chimeric protein, the complimentary DNA sequence encoding the p53 peptide (residues 359-368) was added into the sequence of the 3' primer used for the PCR reaction of the HAUSP TRAF-like domain (residues 53-199). A similar strategy was adopted to generate the HAUSP-MDM2 chimeric protein, in which the MDM2 peptide (residues 223-232) was fused to the C-terminus of the HAUSP N-terminal domain (residues 53-197). Both chimeric proteins were cloned into the vector pGEX-2T (Pharmacia) and were overexpressed in BL21(DE3). All proteins were purified to homogeneity as described (Hu et al., 2002, Cell 111:1041-1054).

[0117] Crystallization and Structure Determination of the HAUSP TRAF-like Domain.

[0118] Crystals were grown by the hanging drop vapor diffusion method by mixing the HAUSP protein (residues 53-206; about 25 milligrams per milliliter) with an equal volume of reservoir solution containing 100 millimolar Tris (pH 8.0), 240 millimolar $CaCl_2$, 20 millimolar $(NH_4)_2SO_4$, and 6.5% PEG4000 (w/v), at 12 degrees Celsius. Small crystals appeared after 2-3 days and continued to grow to full-size in 3-4 weeks. The crystals belong to the space group $P2_12_12_1$ and contain one molecule per asymmetric unit. The unit cell has a dimension of a = 45.17 Angstrom, b = 52.24 Angstrom, c = 63.65 Angstrom. Crystals were equilibrated in a cryoprotectant buffer containing reservoir buffer plus 25% glycerol (v/v) and were flash frozen in a cold nitrogen stream at -170 degrees Celsius. The native and MAD data sets were collected at NSLS beamlines X-25 and X-12C, respectively. All X-ray diffraction data described in this manuscript were processed using the software Denzo and Scalepack (Otwinowski et al., 1997, Methods Enzymol. 276:307-326). The structure was determined by multiple anomalous dispersion (MAD), using SOLVE (Terwilliger et al., 1996, Acta Crystallogr. D52:749-757), and the selenium sites were refined using MLPHARE (Collaborative Computational Project ,1994, Acta Crystallogr. D50:760-763).

All atomic models described in this study were built using O (Jones et al., 1991, Acta Crystallogr. A47:110-119) and refined using CNS (Brunger et al., 1998, Acta Crystallogr. D54:905-921).

[0119] Crystallization and Structure Determination of the HAUSP-p53 Chimeric Protein.

[0120] Crystals were grown by the hanging drop vapor diffusion method by mixing the protein (about 20 milligrams per milliliter) with an equal volume of reservoir solution containing 100 millimolar Tris (pH 7.5), 200 millimolar $CaCl_2$, and 9% (w/v) PEG2000 monomethylether. The crystals belong to the space group C2, and contain two molecules per asymmetric unit. The unit cell has a dimension of a = 87.76 Angstrom, b = 39.56 Angstrom and c = 101.9 Angstrom, and c = 105.3, and contain two molecules per asymmetric unit. The native data set was collected at NSLS X-25. The structure was determined by molecular replacement using AMoRe (Navaza ,1994, Acta Crystallogr. A50:157-163) and refined at 2.3 Angstrom resolution.

[0121] Crystallization and Structure Determination of the HAUSP-MDM2 Chimeric Protein.

[0122] Crystals were grown by the hanging drop vapor diffusion method by mixing the protein (about 15 milligrams per milliliter) with an equal volume of reservoir solution containing 100 millimolar Tris (pH 8.5), 300 millimolar $CaCl_2$, and 26% (w/v) PEG4000. The crystals belong to the space group $P3_221$ and contain one molecule per asymmetric unit. The unit cell has a dimension of a = 37.53 Angstrom, b = 37.53 Angstrom, and c = 177.3 Angstrom. The native data set was collected at NSLS X-25. The structure was determined by molecular replacement using AMoRe (Navaza, 1994, Acta Crystallogr. A50:157-163) and refined at 1.7 Angstrom resolution.

[0123] Crystallization and Structure Determination of HAUSP (Residues 53-560).

[0124] Crystals were grown by the hanging drop vapor diffusion method by mixing the protein (about 15 milligrams per milliliter) with an equal volume of reservoir solution containing 100 millimolar Phosphate - Citrate (pH 4.2), 0.8% PEG 10000, and 50 millimolar 1,6-Hexandiol. Small crystals appeared after two days and were used as seeds to

generate larger crystals. The crystals belong to the space group $C222_1$, and contain two molecules per asymmetric unit. The unit cell has a dimension of a = 97.63 Angstrom, b = 219.9 Angstrom, and c = 130.5 Angstrom. The native data set was collected at CHESS-A1. The structure was determined by molecular replacement using AMoRe (Navaza ,1994, Acta Crystallogr. A50:157-163) and refined at 3.2 Angstrom resolution.

**[0125]** In Vitro Binding Assays Using Gel Filtration

**[0126]** Size exclusion chromatography (gel filtration), using a SUPERDEX-200 column (10/30, Amersham Pharmacia Biotech), was employed to carry out all the in vitro binding assays. Proteins subjected to binding tests were incubated at 4 degrees Celsius for at least 45 minutes to allow equilibrium to be reached. The flow rate was 0.5 milliliters per minute, and the buffer contained 25 millimolar Tris (pH 8.0), 100 millimolar NaCl, and 2 millimolar DTT. All fractions were collected at 0.5 milliliter each. Aliquots of relevant fractions were mixed with SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The proteins were visualized by Coomassie Blue staining. The column was calibrated with molecular mass standards. For competition experiments, a 10-fold molar equivalent of the p53 peptide was first incubated with the HAUSP TRAF-like domain for 5 minutes at 4 degrees Celsius, then the complex was further incubated with one molar equivalent of MDM2 peptide for 45 minutes at 4 degrees Celsius before the mixture was subjected to gel filtration analysis.

**[0127]** Isothermal Titration Calorimetry (ITC).

**[0128]** HAUSP N-terminal domain (residues 53-206), the p53 peptide (residues 351-382), and the MDM2 peptide (residues 208-242) were prepared in the buffer containing 10 millimolar HEPES (pH 8.0) and 100 millimolar NaCl. The Micro Calorimetry System (MICROCAL, Amherst, MA) was used to perform the ITC measurements for the interaction between the HAUSP N-terminal domain and the peptides. The titration data were collected at 4 degrees Celsius and analyzed using the ORIGIN data analysis software (Microcal Software, Northampton, MA).

**[0129]** The results of the experiments presented in this example are now described.

[0130] Structure of the HAUSP TRAF-like Domain

[0131] The p53-binding element of HAUSP to its N-terminal residues 53-208 shares significant homology to the TRAF (tumor necrosis factor-receptor associated factor) domain (Hu et al., 2002, Cell 111:1041-1054; Zapata et al., 2001, J. Biol. Chem. 276:24242-24252). We crystallized this domain (residues 53-206) and determined its structure at 1.65 Angstrom resolution using multi-wavelength anomalous dispersion (results shown in Table 2 and Figure 4A). The HAUSP TRAF-like domain adopts an eight-stranded anti-parallel (beta)-sandwich structure, with strands (beta)1, (beta)5, (beta)6, and (beta)8 in one sheet and strands (beta)2, (beta)3, (beta)4, and (beta)7 in the other (Figure 4A). Strands (beta)7 and (beta)8 are connected by a short (alpha) helix. The (beta)7 strand appears twisted and contains a (beta)-bulge in the middle of the strand.

[0132] The topology and overall structure of the HAUSP TRAF-like domain closely resemble the TRAF-C domain of the TRAF family of proteins, with a root-mean-square deviation (RMSD) of 1.48 Angstrom for 101 C(alpha) atoms compared to TRAF2 (Ni et al., 2000, Proc. Natl. Acad. Sci. USA 97:10395-10399; Park et al., 1999, Nature 398:533-538; Ye et al., 2002, Nature 418:443-447; Li et al., 2002, Structure (Camb) 10:403-411; Ye et al., 1999, Mol. Cell 4:321-330). The (beta) bulge in strand (beta)7 of the HAUSP TRAF-like domain is present in all structures of TRAF-C domains and has been shown to be involved in receptor peptide recognition by TRAFs. In addition, the HAUSP TRAF-like domain contains a shallow surface groove in the middle of the (beta)-sandwich (as shown in Figure 4A), which corresponds to the region where the receptor peptides bind.

[0133] Despite overall structural similarity, amino acid composition in the putative substrate-binding groove of HAUSP differs significantly from that in TRAF2 (as shown in Figure 4B). For example, a set of peptide-interacting residues in TRAF2, including Arg393, Tyr395, Asp399, Phe447, Ser453, Ser454, Ser455, and Ser467, are highly conserved among TRAF1, 2, 3, and 5. However, most of these conserved residues are no longer retained in the HAUSP TRAF-like domain, suggesting different peptide-binding specificity. Moreover, the residues mediating peptide recognition in TRAF6, Arg392, Phe471, and Tyr473, are also missing in the HAUSP N-terminal domain structure. These observations indicate that the HAUSP TRAF-like domain represents a new type of peptide-binding motif in the TRAF family.

[0134] Structural basis of p53 recognition by HAUSP

[0135] The HAUSP TRAF-like domain stably interacts with a C-terminal peptide fragment (residues 357-382) of p53. Deletion of 11 amino acids (residues 357-367) in p53 results in a complete loss of interaction with HAUSP (Hu et al., 2002, Cell 111:1041-1054). Using an in vitro interaction assay, we performed mutagenesis to further define the HAUSP-binding element in p53 (shown in Figure 5A). All mutant p53 fragments were individually purified to homogeneity and were examined for binding to HAUSP using a gel-filtration-based assay. The p53 fragment (residues 325-363) formed a stable complex with HAUSP. Mutations of residues 357, 358, and 363 in p53 (residues 325-363) did not affect the ability of p53 (residues 325-367) to interact with HAUSP. In contrast, mutation of Pro359, Gly361, or Ser362 in p53 (residues 325-363), resulted in abrogation of its interaction with the HAUSP TRAF-like domain. These observations indicate that the minimal HAUSP-binding element is within amino acids 359-363 of p53.

[0136] To elucidate the mechanism by which p53 recognizes HAUSP, we launched rigorous trials aimed at crystallizing the HAUSP TRAF-like domain bound to a synthetic p53 peptide. However, such effort did not yield crystals that are suitable for X-ray diffraction studies. To facilitate formation of a stable p53-HAUSP complex, we generated a chimeric protein with the C-terminus of the HAUSP TRAF-like domain (residues 53-199) fused to 10 amino acids corresponding to p53 residues 359-368. We reasoned that such design would ensure a 1:1 stoichiometry between HAUSP and the p53 peptide. Indeed this engineering effort proved effective and diffraction-quality crystals were successfully obtained. The structure of the HAUSP TRAF-like domain bound to p53 (residues 359-368) was determined at 2.3 Angstrom resolution by molecular replacement (results are shown in Table 3 and Figure 5B).

[0137] The p53 peptide binds to the shallow surface groove near one edge of the (beta)-sandwich in the TRAF-like domain (as shown in Figure 5B). Only four contiguous amino acids of p53, Pro359-Gly360-Gly361-Ser362, make specific interactions to residues in HAUSP. Arg363 adopts a well-defined conformation but is not directly involved in HAUSP recognition (see Figure 5C). At the N-terminus, the side chain of Pro359 is nestled in a hydrophobic pocket at the edge of the (beta)-sandwich, which is formed by the backbone C(alpha) atom of Gly166 and the hydrophobic side chains of Phe167, Trp165, and Ile154. At the C-terminus, the carboxylate side chain of Asp164 accepts two hydrogen

bonds from the amide and the hydroxyl groups of Ser362. In addition, the side chain of Ser362 makes van der Waals contacts to Phe118. In the center of the bound p53 peptide, the amide and carbonyl groups of Gly360 are hydrogen bonded to the main chain groups of Gly166 from HAUSP (Figure 5C). The main chain C(alpha) of Gly360 makes van der Waals contacts to Trp165 in HAUSP. Residues 364-368 of p53 are disordered in the crystals and do not contribute to HAUSP binding.

**[0138]** The structural observations are fully consistent with our mutagenesis results. For example, Pro359 and Ser362 in the p53 peptide anchor its interaction with the HAUSP TRAF-like domain; missense mutation of Pro359 or Ser362 in p53 abolished its binding to HAUSP. To further corroborate the structural findings, we also performed additional mutagenesis on the HAUSP TRAF-like domain. Mutation of Trp165 and Phe167 in HAUSP, both involved in the hydrophobic pocket that accommodates Pro359 of p53, resulted in a complete loss of binding to p53. Similarly, mutation of Asp164, which forms hydrogen bonds with S362 of p53, also significantly weakened the ability of HAUSP to bind to p53.

**[0139]** Mapping HAUSP-MDM2 interactions

**[0140]** MDM2 is known to specifically associate with HAUSP and represents a physiological substrate for HAUSP-mediated deubiquitinating activity. To elucidate the mechanism by which HAUSP recognizes MDM2, we first sought to identify the minimal domain in HAUSP that is responsible for binding to MDM2 and the results are summarized in Figure 6A. Surprisingly, the N-terminal TRAF-like domain of HAUSP (residues 1-206) was found to form a stable complex with MDM2 (residues 170-423). This result indicates that the same domain of HAUSP that interacts with p53 is also responsible for binding to MDM2. Neither the central isopeptidase domain nor the C-terminal extension of HAUSP was required for binding to MDM2 (residues 170-423).

**[0141]** Next, we mapped the minimal HAUSP-binding element in MDM2 and the results are summarized in Figure 6B. The full-length human MDM2 (known as HDM2), containing 491 amino acids, can be divided into four major conserved regions: an N-terminal domain responsible for binding to p53, a highly acidic region that is involved in binding to multiple proteins, a putative Cys4-type zinc finger domain, and a RING finger domain responsible for the E3 ubiquitin ligase activity of MDM2 (Oliner et al., 1992,

Nature 358:80-83). We first found that a 33-kDa fragment of MDM2 (residues 170-432) formed a stable complex with the full-length HAUSP as well as the HAUSP N-terminal TRAF-like domain. Next, we generated a series of deletion variants of MDM2 and assayed their interactions with the HAUSP TRAF-like domain using a gel-filtration assay. Neither the central acidic region nor the zinc finger domain of MDM2 interacted with HAUSP (residues 53-206). MDM2 fragments encompassing either residues 223-289 or residues 170-232 formed stable complexes with HAUSP TRAF-like domain (residues 53-206), suggesting an essential role of amino acids 223-232 in MDM2. Supporting this notion, the 10-amino acid MDM2 fragment (residues 223-232) was found to form a stable complex with the HAUSP TRAF-like domain.

**[0142]** Mutually Exclusive HAUSP Binding by p53 and by MDM2

**[0143]** The observations that both a p53 C-terminal peptide fragment and a short peptide sequence preceding the acidic domain of MDM2 bind to the N-terminal TRAF-like domain of HAUSP suggested that HAUSP binding by p53 and by MDM2 might be mutually exclusive. To test this hypothesis, we performed an in vitro competition assay using gel filtration chromatography. The HAUSP TRAF-like domain (residues 1-206) was incubated with one molar equivalence of MDM2 (residues 208-289) in the presence of 10 molar equivalents of p53 (residues 351-382) and then the mixture was subjected to a gel filtration analysis. Despite a 10-fold excess of the p53 peptide over MDM2, the MDM2 fragment, but not the p53 peptide, formed a stable complex with HAUSP (residues 1-206). This result suggests that MDM2 binds to HAUSP with a higher affinity than p53.

**[0144]** To assess the strength of HAUSP binding by p53 and by MDM2, we measured the binding affinity between the HAUSP TRAF-like domain (residues 53-206) and a p53 peptide (residues 351-382) or a MDM2 peptide (residues 208-242) using isothermal titration calorimetry (ITC). Under identical experimental conditions, the MDM2 peptide bound to the HAUSP TRAF-like domain with a dissociation constants of 3 μM, 7-fold tighter than that of the p53 peptide. Thus these experiments confirm the competitive binding edge of MDM2 over p53. The observed binding affinity between the p53 peptide and the HAUSP TRAF-like domain is in agreement with a previous report (Saridakis et al., 2005, Mol. Cell 18:25-36). It should be noted that the binding affinity of the full-length MDM2 is likely higher than the value reported here, because the full-length MDM2 protein contains at least

two more binding sites for HAUSP and the multiple binding sites serve to significantly increase the effective protein concentration.

[0145] Structural basis of HAUSP recognition by MDM2

[0146] Intriguingly, the minimal MDM2 fragment (residues 223-232) required for binding to HAUSP exhibited little sequence homology to the p53 fragment that interacts with the same domain of HAUSP. To elucidate the mechanism by which MDM2 recognizes HAUSP, we sought to determine the structure of HAUSP TRAF-like domain bound to the minimal MDM2 fragment. We engineered a chimeric protein with the MDM2 peptide (residues 223-232) fused to the C-terminus of the HAUSP TRAF-like domain (residues 53-197). We crystallized this chimeric protein and solved its structure at 1.7 Angstrom resolution (results are shown in Table 3 and Figure 7A).

[0147] In the structure of the HAUSP-MDM2 fusion protein, the MDM2 peptide (residues 223-230) is bound to the shallow surface groove on one side of the (beta)-sandwich in the TRAF-like domain -- the same site at which p53 binds. The MDM2 peptide assumes an extended main chain conformation, extending across strands (beta)3, (beta)4, (beta)6, and (beta)7 (as shown in Figure 7A). Five residues in the MDM2 peptide, Asp225-Ala226-Gly227-Val228-Ser229, make extensive polar and non-polar interactions with the surface binding residues from all four strands, particularly strand (beta)7 (as shown in Figure 7B). Although Asp223, Leu224, and Glu230 of MDM2 adopt well-defined conformation in the structure, they do not make direct contribution to interactions with HAUSP.

[0148] At the N-terminal end of the MDM2 peptide, the carboxylate side chain of Asp225 accepts a charge-stabilized hydrogen bond from the guanidinium group of Arg152 in HAUSP. The main chain conformation of Asp225 is stabilized by a pair of intra-molecular hydrogen bonds between the carbonyl group of Asp223 and the amide groups of Asp225 and Ala226. In the center of the MDM2 peptide, three residues make a number of van der Waals interactions to HAUSP. The side chain of Ala226 in MDM2 packs against a hydrophobic pocket formed by the backbone C(alpha) atom of Gly166 and the side chains of Phe167, Trp165, and Ile154 in HAUSP. The main chain groups of Gly227 in MDM2 make two hydrogen bonds to Gly166 in HAUSP whereas the backbone C(alpha) atom of Gly227 is within van der Waals contact distance of the hydrophobic side chain of Trp165 in

HAUSP. The side chain of Val228 in MDM2 interacts with a hydrophobic surface patch formed by the side chain of Trp165 and the aliphatic portion of the side chains of Glu162 and Asp164 in HAUSP. At the C-terminal end of the MDM2 peptide, Ser229 in MDM2 makes three hydrogen bonds to surface residues in HAUSP. The amide and the hydroxyl groups of Ser229 donate two hydrogen bonds to the carboxylate side chain of Asp164 in HAUSP, and the carbonyl group of Ser229 accepts a hydrogen bond from the guanidinium group of Arg104 in HAUSP.

**[0149]** Comparison of HAUSP Binding by MDM2 and by p53

**[0150]** To compare the specific recognition of p53 and MDM2 by HAUSP, we superimposed the structure of the HAUSP TRAF-like domain bound to p53 peptide to the structure of the TRAF-like domain bound to MDM2. This alignment resulted in an RMSD of 0.37 Angstrom for 134 aligned backbone C(alpha) atoms, with the bound p53 and MDM2 peptides well superimposed to each other and is shown in Figure 8A.

**[0151]** There are a number of common themes for the recognition of p53 and MDM2 by HAUSP. Both p53 and MDM2 peptides bind to the same surface groove in the HAUSP TRAF-like domain, sharing similar overall orientation (compare Figures 8A and 8B). Two non-polar residues, Pro359 in p53 and Ala226 in MDM2, are positioned at the same site upon binding to HAUSP and make similar van der Waals contacts with a hydrophobic surface pocket near the edge of (beta)-sandwich in HAUSP. The residues following Pro359 in p53 and Ala226 in MDM2 are both glycines, which are very well superimposed and make conserved main chain hydrogen bonds to Gly166 in the (beta)7 strand of HAUSP (see Figures 8B and 8C). Another conserved residue in both peptides is Ser362 in p53 and Ser229 in MDM2, which in both cases make hydrogen bonds to Asp164 in HAUSP and make van der Waals contacts to Phe118 in HAUSP. The multiple specific interactions between this Ser residue and residues from HAUSP suggest that this Ser plays an anchoring role in the specific recognition of both p53 and MDM2 by HAUSP.

**[0152]** Despite these common features, important differences exist for HAUSP binding by p53 and by MDM2, which explain the competitive binding of MDM2 relative to p53. Gly361 in p53, which does not directly interact with HAUSP, is substituted by Val228 in MDM2, resulting in additional van der Waals contacts with Trp165 and Glu162 in HAUSP (see Figure 7B). Perhaps more significantly, Asp225 in MDM2 hydrogen bonds with

Arg152 and Ser168 in HAUSP. Such interactions are absent in the case of bound p53. Compared to p53, the more extensive interactions between MDM2 and HAUSP provide a mechanistic explanation to our biochemical observation that MDM2 out-competed excess p53 for binding to HAUSP.

[0153] Peptide Recognition by HAUSP

[0154] In this example, the structural basis for the differential recognition of p53 and MDM2 by HAUSP is described. HAUSP was previously known to bind to another protein named EBNA1 , and the crystal structure of the HAUSP TRAF-like domain bound to a peptide derived from EBNA1 was recently reported (Saridakis et al., 2005, Mol. Cell 18:25-36; Holowaty et al., 2003, J. Biol. Chem. 278:47753-47761; Holowaty et al., 2003, J. Biol. Chem. 278:29987-29994). The amino acid sequences of the three HAUSP-binding peptides, PGGSR in p53, DAGVSE in MDM2, and PGEGPS in EBNA1 share no obvious sequence homology with each other; yet they all bind to the same surface groove on the TRAF-like domain of HAUSP (Figure 8B). Comparison of these three structures reveals how HAUSP specifically recognizes all three distinct peptides.

[0155] Structure-based sequence alignment of the three HAUSP-binding peptides revealed a conserved four-residue binding motif with the consensus sequence of ({phi}/E)-G-({phi}/G)-S, where {phi} denotes a non-polar residue (see Figure 8C). In all three structures, the four-residue motif, in an extended conformation, binds to the same substrate recognition site on HAUSP and make conserved contacts with the protein (as shown in Figure 8B). The first residue P1 in the binding motif is either a non-polar residue such as Pro359 in p53 or Ala226 in MDM2, or Glu444 as in EBNA1 which also has an aliphatic portion in its side chain. P1 makes van der Waals interactions with a hydrophobic surface pocket on one edge of the (beta)-sandwich in HAUSP. P2, an invariant Gly in all three peptides, forms two main chain hydrogen bonds with Gly166 in the (beta)7 strand of HAUSP. P3, a hydrophobic residue in MDM2 and EBNA1, stacks against a small hydrophobic surface patch on HAUSP. This residue is Gly in p53, resulting in a loss of van der Waals interaction at the P3 position and consequently contributing to a relatively low binding affinity. P4, an invariant Ser in all three peptides, makes two hydrogen bonds to the carboxylate side chain of Asp164 in HAUSP and contacts Phe118 from (beta)4 strand of

HAUSP. This analysis identifies Ser at the P4 position as the anchoring residue for specific binding of peptides to HAUSP.

[0156] The residues outside the central HAUSP-binding motif are diverse, both in terms of their corresponding positions and in terms of interactions they mediate. Both MDM2 and EBNA1, but not p53, contain extra HAUSP-interacting residues at the N-terminal end of the motif. In EBNA1 peptide, the N-terminal Pro442 and Gly443 run parallel to the edge of the HAUSP (beta)7 strand, forming hydrogen bonds between the carbonyl group of Asp441 and the side chain of Asn169 in HAUSP and between the carbonyl group of EBNA1 Pro442 and the amide group of Ser168 in HAUSP. Moreover, Pro442 has additional hydrophobic contacts with Phe167 in HAUSP. These additional interactions are very different from those between MDM2 and HAUSP, but explain why EBNA1 out-competes p53 for binding to HAUSP.

[0157] The fact that most of the conserved peptide-binding residues in TRAFs are not present in the structure of the HAUSP TRAF-like domain indicates that the TRAF-like domain of HAUSP represents a new peptide-binding motif in the TRAF family. Supporting this notion, the minimal HAUSP-binding motif does not match any of the known TRAF-binding sequences, including the major consensus sequence of (P/S/A/T)X(Q/E)E and the minor consensus sequence of PXQXXD for TRAF1, 2, 3, and 5, and the TRAF6 binding motif of PxExx({phi}/acidic).

[0158] Coordination of Substrate Recognition and Deubiquitination by HAUSP

[0159] HAUSP is a representative member of the UBP family of DUBs, which are known to recognize specific substrates for deubiquitination. The recognition of a substrate is expected to be coupled to its deubiquitination by HAUSP. However, there was previously a complete lack of understanding for this process. To investigate this process, we sought to determine the crystal structure of a HAUSP fragment (residues 53-560), which contains both the substrate-binding TRAF-like domain and the catalytic core domain. We generated crystals for this HAUSP fragment and solved its structure at 3.2 Angstrom resolution (results shown in Table 3). The structure contains two well-ordered domains, the TRAF-like domain as a (beta)-sandwich and the isopeptidase domain as a tri-partite architecture (Figure 9A). There is very limited interaction between these two domains and the intervening linker sequences (residues 199-206) have high temperature factors in the

crystals, suggesting that the substrate-binding domain is coupled to the catalytic domain through a relatively flexible linker sequence. The ubiquitin-binding pocket of the catalytic core domain and the substrate-binding groove of the TRAF-like domain are located on the same side of the molecule (see Figure 6).

**[0160]** On the basis of the structure and previous information about ubiquitin binding (Hu et al., 2002, Cell 111:1041-1054), we modeled how HAUSP may recognize a ubiquitinated MDM2 molecule. The results are shown in Figure 9B. In this model, a small peptide region (residues 225-230) of MDM2 anchors its binding to the TRAF-like domain of HAUSP. A conjugated ubiquitin moiety is bound by the Fingers domain of HAUSP, with the C-terminus of ubiquitin docked in the active site cleft between the Palm and the Thumb. The close proximity of the C-terminus of HAUSP-bound ubiquitin and the MDM2 peptide suggests that HAUSP prefers to cleave the ubiquitin chain at the proximal end of the ubiquitinated MDM2.

**[0161]** Conclusions that can be drawn from the experiments presented in this example are now described.

**[0162]** The critical role of HAUSP in regulating the p53-MDM2 pathway was first revealed by the observations that HAUSP can specifically recognize and deubiquitinate p53 both in vivo and in vitro (Li et al., 2002, Nature 416:648-653). However, the concept of HAUSP as a specific p53-stabilizing protein and candidate tumor suppressor was challenged by the recent observations that HAUSP also specifically recognizes MDM2 and stabilizes MDM2 through direct deubiquitination (Cummins et al., 2004, Cell Cycle 3:689-692; Cummins et al., 2004, Nature 428:1 p following p. 486). Based on those reports, a dynamic role of HAUSP in the p53-MDM2 pathway was proposed (Li et al., 2004, Mol. Cell 13:879-886). However, the mechanisms by which HAUSP recognizes p53 and MDM2, as well as the role of HAUSP in the p53-MDM2 pathway, remained unknown.

**[0163]** In this disclosure, we report at least four important and novel findings. First, the N-terminal TRAF-like domain of HAUSP/USP7 has been found to specifically recognize both p53 and MDM2. This conclusion indicates that the substrate recognition domain of a UBP (such as HAUSP) can target more than one cellular protein. Second, MDM2 binding to HAUSP was found to be mutually exclusive with p53 binding to HAUSP. The HAUSP-binding elements were mapped to a peptide fragment in the carboxyl-terminus of p53 and to

a short peptide region preceding the acidic domain of MDM2. A minimal HAUSP-binding peptide derived from MDM2 efficiently displaced p53 from the p53-HAUSP complex in a competition assay and formed a stable MDM2-HAUSP complex. Third, the molecular basis of HAUSP-mediated recognition of p53 and MDM2 was revealed by high-resolution crystal structure determinations of the HAUSP TRAF-like domain bound to peptides derived from p53 and MDM2. Structural comparison reveals that MDM2 recognizes the same surface groove in the HAUSP TRAF-like domain as that recognized by the p53 peptide. Compared to p53, MDM2 makes conserved yet more extensive contacts with HAUSP. Fourth, structural analysis revealed a consensus peptide sequence for HAUSP recognition. This consensus sequence greatly facilitates any effort aimed at identifying additional substrates for HAUSP/USP7. For example, Hdmx, a MDM2 homologue, was shown to bind to HAUSP (Meulmeester et al., 2005, Mol. Cell 18:565-576); examination of the primary sequences of Hdmx identified a consensus HAUSP-binding peptide $A_{470}GAS_{473}$. This structural analysis also identifies the TRAF-like domain in HAUSP as a new class of peptide-binding TRAF domains.

**[0164]** This disclosure provide an important framework for understanding the function of HAUSP in the p53-MDM2 pathway. Although HAUSP can bind with p53, our competition data suggest that HAUSP exhibits greater binding affinity for MDM2, which likely translates into a stronger preference for MDM2 deubiquitination and stabilization in a physiological setting. This conclusion further suggests that MDM2 is a preferred substrate for HAUSP activity under physiological conditions and that HAUSP has a critical role in antagonizing the auto-ubiquitination function of MDM2. This analysis is fully consistent with the observation that p53 is stabilized but MDM2 is destabilized in HAUSP-ablated cells.

**[0165]** For a protein such as MDM2 or p53, ubiquitination frequently occurs to multiple Lys residues that are spread out in the primary sequences and in space. It was previously unknown how a UBP can effectively deubiquitinate a protein at multiple sites. This disclosure provides such a mechanism. First, a protein can contain multiple binding sites for the corresponding UBP. Supporting this notion, a consensus HAUSP-binding element has been described which contains only four amino acid residues, ({phi}/E)-G-({phi}/G)-S. The small size of this element indicates a statistically reasonable chance for finding multiple sites in a single protein. Examination of the complete human MDM2 sequence revealed two

additional candidate HAUSP-binding elements, $D_{117}SGTS_{121}$ and $L_{129}EGGS_{133}$. The presence of multiple HAUSP recognition sites in a given protein can greatly facilitate efficient deubiquitination. Second, the linker sequence between the TRAF-like domain and the catalytic domain is flexible, which allows lateral relative movement between these two domains, enhancing access to multiple ubiquitination sites within the same substrate.

**[0166]** It is worth noting that the consensus peptide sequence for HAUSP binding includes a serine residue, which can be phosphorylated, as an anchoring residue. This residue makes a conserved hydrogen bond to the negatively charged Asp164 in p53 (see Figure 5), in MDM2 (see Figure 7), and in EBNA1. Structural analysis indicates that phosphorylation of this Ser residue negatively impacts its ability to contribute to HAUSP binding. Thus, phosphorylation of Ser362 in p53 and Ser229/Ser133/Ser121 in MDM2 can have an important role in regulation of their stability in vivo.

**[0167]** The information in this disclosure is significant for design of pharmaceutical agents for modulating interaction of HAUSP with other proteins. Since HAUSP plays an essential role in the stabilization of MDM2, negative regulation of HAUSP can suppress MDM2 activity, which in turns stabilizes the tumor suppressor protein p53. This disclosure identifies differential features for HAUSP binding by MDM2 and by p53. For example, MDM2 contains an additional binding residue (Asp225) that makes contacts to an area of HAUSP that is irrelevant for p53 binding. These different binding characteristics can be used to design compounds to specifically inhibit binding of HAUSP with one or both of MDM2 and p53. Because complete inhibition of HAUSP is likely to have a far greater negative impact on MDM2 over p53, the structural information provided herein for the entire surface groove of HAUSP TRAF-like domain can be used to design and test (e.g., in a computer simulation or in a biochemical assay such as one of those described herein) compounds for inhibiting or preventing interaction of any protein with the domain. The high-resolution structures described herein greatly facilitate design and modeling of such compounds. Such design and screening efforts can be carried out in conjunction with inhibitor screening aimed at suppressing the isopeptidase activity of HAUSP.

Table 2. Summary of crystallographic analysis on HAUSP TRAF-like domain.

| Data set | Native HAUSP (53-206) | SeMet λ1 (peak) | SeMet λ2 (Inflection) | SeMet λ3 (Remote) |
|---|---|---|---|---|
| Beamline | NSLS-X25 | NSLS-X12C | NSLS-X12C | NSLS-X12C |
| Wavelength (Å) | 1.1 | 0.9794 | 0.9797 | 0.9500 |
| Resolution (Å) | 99 – 1.60 | 99 – 2.40 | 99 – 2.40 | 99 – 2.40 |
| Unique reflections | 20,180 | 5,801 | 5,768 | 5,667 |
| Data redundancy | 6.5 | 7.1 | 7.0 | 7.0 |
| Completeness, % (Outer shell) | 95.2 (60.0) | 90.1 (48.6) | 89.5 (47.5) | 87.5 (40.4) |
| I/σ (Outer shell) | 36.8 (6.3) | 15.3 (2.9) | 14.4 (2.8) | 13.4 (2.3) |
| $R_{sym}$ (Outer shell) | 0.048 (0.203) | 0.11 (0.31) | 0.099 (0.29) | 0.12 (0.40) |
| Anomal. Diff. (%) | n/a | 7.0 | 6.6 | 7.2 |
| $R_{cullis}$ | | 0.69 | 0.68 | 0.66 |
| Phasing power (centric/acentric) | | 1.57/1.28 | 1.36/1.13 | 1.71/1.36 |
| Overall Figure of Merit (20 – 2.4 Å) | | 0.55 | | |

Refinement statistics:

| Resolution range (Å) | Number of reflections (\|F\| > 0) | Total number of atoms (water) | Completeness of data | R-factor[2] (R-free) | RMSD[3] Bond (Å) | RMSD[3] Angle (deg.) |
|---|---|---|---|---|---|---|
| 20 – 1.65 | 18,198 | 1,335 (174) | 97.4% | 0.194 (0.212) | 0.009 | 1.56 |

Ramachandran plot:

| Most favored (%) | Additionally allowed (%) | Generously allowed (%) | Disallowed (%) |
|---|---|---|---|
| 86.9% | 13.1% | 0.0% | 0.0% |

$R_{sym} = \Sigma_h \Sigma_i \mid I_{h,i} - I_h \mid / \Sigma_h \Sigma_i I_{h,i}$, where $I_h$ is the mean intensity of the $i$ observations of symmetry related reflections of $h$. $R_{cryst} = \sum \mid F_{obs} - F_{calc} \mid / \sum F_{obs}$, where $F_{obs} = F_P$, and $F_{calc}$ is the calculated protein structure factor from the atomic model ($R_{free}$ was calculated with 10% of the reflections). Phasing power = $[(F_{H(calc)})^2 / (F_{PH(obs)} - F_{PH(calc)})^2]^{1/2}$, where $F_{PH(obs)}$ and $F_{PH(calc)}$ are the observed and calculated derivative structure factors, respectively. $R_{Cullis} = \sum \| F_{PH} \pm F_P \mid - F_{H(calc)} \mid / \sum \mid F_{PH} - F_P \mid$, where $F_{H(calc)}$ is the calculated heavy atom structure factor. Figure of Merit = $<\sum P(\alpha) \exp(i\alpha) / \sum P(\alpha)>$, where $P(\alpha)$ is the probability distribution for the phase $\alpha$. RMSD (root-mean-square deviation) in bond lengths and angles are the deviations from ideal values.

Table 3. Summary of crystallographic analysis.

| Data set | HAUSP-p53 | HAUSP-MDM2 | HAUSP (53-560) |
|---|---|---|---|
| Beamline | NSLS-X25 | NSLS-X25 | CHESS-A1 |
| Spacegroup | C2 | $P3_221$ | $C222_1$ |
| Resolution (Å) | 2.30 | 1.70 | 3.20 |
| Unique reflections | 15,182 | 16,752 | 22,308 |
| Data redundancy | 5.8 | 3.9 | 4.5 |
| Completeness, % (Outer shell) | 92.2 (73.6) | 98.0 (92.7) | 95.3 (96.7) |
| I/σ (Outer shell) | 38.3 (14.2) | 15.6 (2.98) | 11.9 (2.79) |
| $R_{sym}$ (Outer shell) | 0.049 (0.151) | 0.086 (0.275) | 0.109 (0.445) |
| Refinement | HAUSP-p53 | HAUSP-MDM2 | HAUSP (53-560) |
| Resolution (Å) | 20.0 – 2.30 | 20.0 – 1.70 | 20.0 – 3.2 |
| Reflections (\|F\|>0) | 13,952 | 28,992 | 20,764 |
| All atoms (solvent) | 2,408 (107) | 1,302 (183) | 7,774 (0) |
| $R_{cryst}/R_{free}$ (%) | 22.7 / 26.3 | 21.8 / 23.7 | 26.5 / 31.6 |
| RMSD bond length (Å) | 0.007 (Å) | 0.005 (Å) | 0.009 (Å) |
| RMSD bond angle (deg) | 1.52 | 1.41 | 1.72 |
| Ramachandran plot: | HAUSP-p53 | HAUSP-MDM2 | HAUSP (53-560) |
| Most favored (%) | 85:5 | 90.2 | 77.7 |
| Additionally allowed (%) | 14.1 | 9.8 | 18.4 |
| Generously allowed (%) | 0.4 | 0 | 3.3 |
| Disallowed (%) | 0 | 0 | 0.6 |

$R_{sym} = \Sigma_h\Sigma_i \mid I_{h,i} - I_h \mid / \Sigma_h\Sigma_i I_{h,i}$, where $I_h$ is the mean intensity of the $i$ observations of symmetry related reflections of $h$. $R_{cryst} = \sum \mid F_{obs} - F_{calc} \mid / \sum F_{obs}$, where $F_{obs} = F_P$, and $F_{calc}$ is the calculated protein structure factor from the atomic model ($R_{free}$ was calculated with 10% of the reflections). RMSD (root-mean-square deviation) in bond lengths and angles are the deviations from ideal values.

[0168] Example 3

[0169] The disclosure of Hu et al., 2002, Cell 111:1041-1054 is referenced and incorporated by reference. That publication described the crystal structure of the isopeptidase domain of HAUSP in isolation and in complex with ubiquitin aldehyde. At the time the Hu et al. reference was published, the physiological role of HAUSP was not well understood. In view of the disclosure made herein, a skilled artisan is able to use the information disclosed in the Hu et al. reference, in combination with the information in this disclosure in the inhibitor design and screening methods described herein. It is thus recognized that at least three types of inhibitors can be designed and made using the information described herein. First, inhibitors which modulate interaction of the TRAF-like domain of HAUSP with proteins with which HAUSP normally binds can be designed and made. Second, inhibitors which modulate interaction of ubiquitin with HAUSP can be designed and made. Third, inhibitors which modulate the isopeptidase activity of HAUSP can be designed and made.

[0170] The disclosure of every patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety.

[0171] While this subject matter has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations can be devised by others skilled in the art without departing from the true spirit and scope of the subject matter described herein. The appended claims include all such embodiments and equivalent variations.